Lab note: Target Part

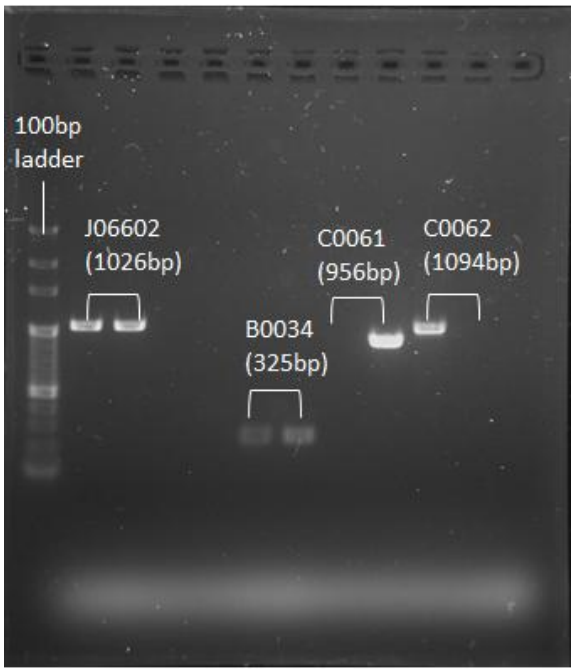
1. First stage: Preparation of assembly template

In order to link each part together to generate the final circuit, we adopt “IGEM standard enzyme cutting sites” to ligate them altogether. Some of the template will be digested first to yield the required cutting sites. On the other hand, some assembly templates would be prepared by using primer PCR to generate the specific cutting sites and the coding sequences.

(1) Igem toolkit transformation (BBa_C0061; BBa_C0062; BBa_J06602)

Transform the toolkit according to the protocol on IGEM official website and do colony PCR.

Colony PCR mix→PCR RUN→Gel check
Results:



Two colonies of BBa_J06602, one colony of BBa_C0061, one colony of BBa_C0062 are correct.

(2) Liquid culture (BBa_C0061; BBa_C0062; BBa_J06602)

a. Pick the colonies which sizes are confirmed from the miniprep second time plates in the laminar flow.

b. Add each colony into a 15mL centrifuging tube with 5mL LB broth.
c. Overnight culture in 37 °C.

(3) Plasmid extraction (BBa_C0061; BBa_C0062; BBa_J06602)

a. Results:
BBa_C0061: 178.03ng/uL
BBa_C0062:172.05ng/uL
BBa_J06602:213.87 ng/uL

(4) **Igem toolkit transformation (BBa_R0062) Gel check**

Results: The sizes of all of the four colonies we pick are all correct.

(5) Plasmid extraction (BBa_R0062)

2. Second stage: Primer PCR for adding specific enzyme cutting sites onto the coding sequence.

Primer design overview figure:

E.coli						
Promoter+RBS		Length		Tm	GC%	Product size
FP	VF2					73
RP	agctgtttcctgtgtgaaattg	22	22	55	41	
LuxR						
FP	atgaaaaacataaatgccgacga	23	23	55	35	
RP	gtgatctacactagcactatcagt	24	24	55	42	781
Terminator+Spe1 site						
FP	ccaggcatcaaataaaacgaaag	23	23	54	39	
RP	GCTACTAGTtataacgcagaaaggccc	28	19	53	47	138
S.mutans						
EcoR1 site +Constitutive promoter+RBS+spacer		Length		Tm	GC%	Product size
FP	CCGGAATTC TTGTTTTATTATTAGAAAGGTGTTACAATTATAACG	45	36	56	22	
RP	TAGGAACCTCCAAATTTTAAACTGT	25	25	55	32	498
LuxI+Spe1 site						
FP	atgactataatgataaaaaaatcggatttttg	33	33	55	21	
RP	GCTACTAGTgagatctacactagcactatcagag	34	25	55	44	652
Terminator+BamH1 site						
FP	ccaggcatcaaataaaacgaaa	22	22	54	36	
RP	TGTGGATCCtataaacgcagaaaggccca	29	20	56	45	

(1) Primer Taq PCR(BBa_C0061; BBa_C0062)

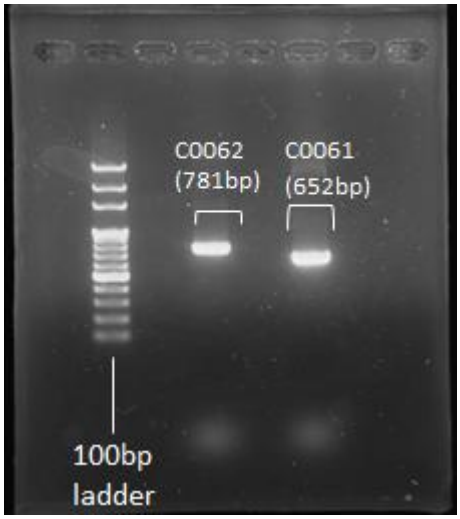
(a) PCR mix:

- 10X Dream Taq Buffer: 2.5uL
- 10mM dNTP: 0.5uL
- Primer:0.5uL(f+r)
- Template:0.5uL
- Dream Taq polymerase: 0.2uL
- ddH_2O: 20.8uL

Total volume: 25uL

Taq PCR →PCR RUN→Gel check

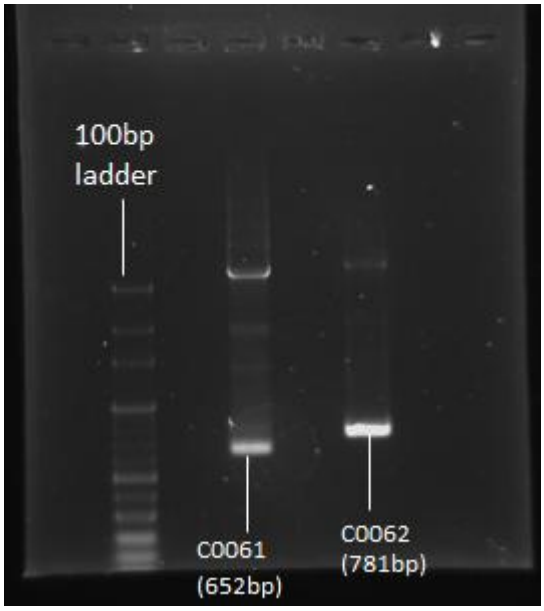
Results:



(2) Primer KOD PCR(BBa_C0061; BBa_C0062)

With the correct sizes confirmed by Gel electrophoresis, we run the KOD PCR with the same templates and primers yet adopt different polymerase, KOD polymerase, that is capable of proofreading during the period of DNA replication.

KOD PCR →PCR RUN→Gel check
Results:



With the correct result of KOD PCR, we load the rest 20uL of the PCR product into the 1.2% Gel, and run the gel electrophoresis again then do the gel extraction protocol.

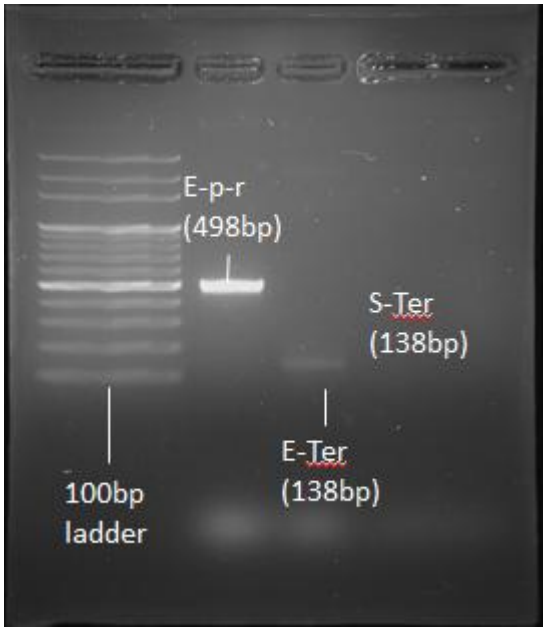
a. Results:

BBa_C0062:88.29 ng/uL
BBa_C0061:12.4ng/uL

(3) Primer Taq PCR(E-p-r; E-ter; S-ter)

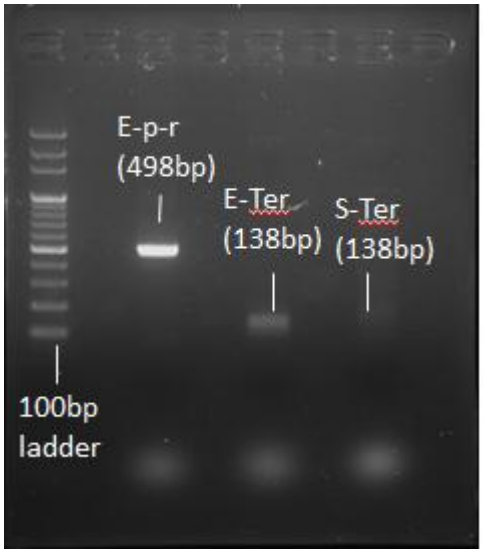
E-p-r stands for promoter plus RBS of E.coli. The template we use is the plasmid DNA of BBa_K523013.
E-ter stands for terminator of *E.coli*. The template we use is the plasmid DNA of BBa_B0015.
S-ter stands for terminator of *S.mutans*. The template we use is the plasmid DNA of BBa_B0015.

(a)Gel electrophoresis check:



With the correct sizes of “E-p-r” and “E-ter” confirmed by Gel electrophoresis, we run the KOD PCR with the same templates and primers yet adopt different polymerase, KOD polymerase, that is capable of proofreading during the period of DNA replication. Meanwhile, we re-run Taq PCR of S-ter using the same PCR program.

Gel electrophoresis check:



With the correct result of KOD PCR(E-p-r and E-ter), we load the rest 20uL of the PCR product into the 1.2% Gel, and run the gel electrophoresis again then do the gel extraction protocol.
Results:
E-p-r:60.68ng/uL
E-ter: 76.36ng/uL

(4) Primer Taq PCR(S-p-r; E-p-r; S-ter)

In order to get better results of the PCR reaction, we make trials on different concentration of Mg^{2+}.
Besides, the “S-p-r” stands for the promoter and RBS part of *S.mutans* and “S-ter” stands for terminator of *S.mutans*.

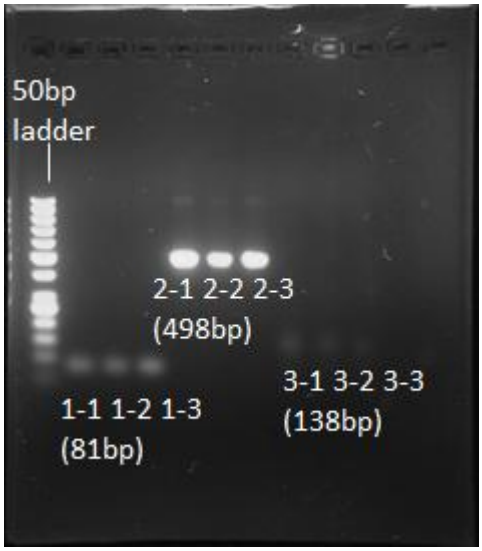
(a) PCR mix:

Unit (uL)	S-p-r(1-1)	S-p-r(1-2)	S-p-r(1-3)	E-p-r(2-1)	E-p-r(2-2)	E-p-r(2-3)	S-ter(3-1)	S-ter(3-2)	S-ter(3-3)
10xDream taq buffer	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5

10mMdNTP	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Primer	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Template	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Dream Taq polymerase	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
25mM Mg^{2+}	0.5	1	1.5	0.5	1	1.5	0.5	1	1.5
ddH_2O	20.4	19.9	19.4	20.4	19.9	19.4	20.4	19.9	19.4
Total volume	25	25	25	25	25	25	25	25	25

Taq PCR →PCR RUN→Gel check

Results:



(5) Primer KOD PCR(S-p-r; S-ter;)

With the correct sizes confirmed by Gel electrophoresis, we run the KOD PCR with the same templates and primers yet adopt different polymerase, KOD polymerase, that is capable of proofreading during the period of DNA replication.

Due to the gel check results of Taq PCR, we choose proper Mg^{2+} concentration of each to run the KOD PCR.

(6) Gel extraction(S-p-r; S-ter)

With the correct result of KOD PCR, we load the rest 20uL of the PCR product into the 1.2% Gel, and run the gel electrophoresis again then do the gel extraction protocol.

a. Results:

S-p-r: 38.73ng/uL

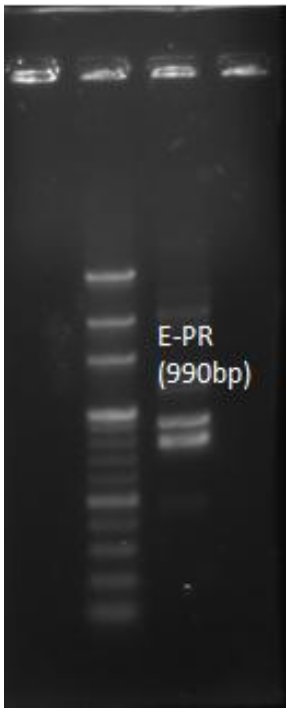
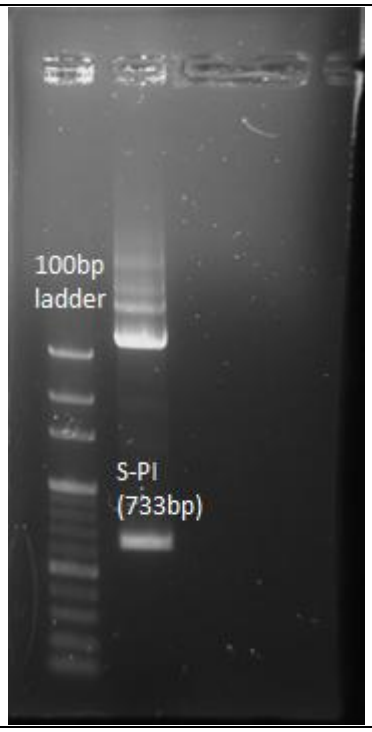
S-ter:64.36ng/uL

3. Third stage: Assembly the elements of circuits together

(1) E-p-r+E-luxR/ S-p-r+S-luxI junction PCR

By designing junction primers, we respectively connect the E-coli promoter region (E-p-r) with the luxR coding gene and the S.mutans promoter region with the luxI coding gene.

Junction PCR →PCR RUN→Gel check

E-PR(E-p-r+luxR)	S-PI(S-p-r+luxI)
	

We slice the 990bp band from the agarose gell and do gel-extrction to obtain the

assembly product of E-PR(E.coli promoter+luxR gene) and S-PI(S.mutans promoter +luxI)

Concentration:

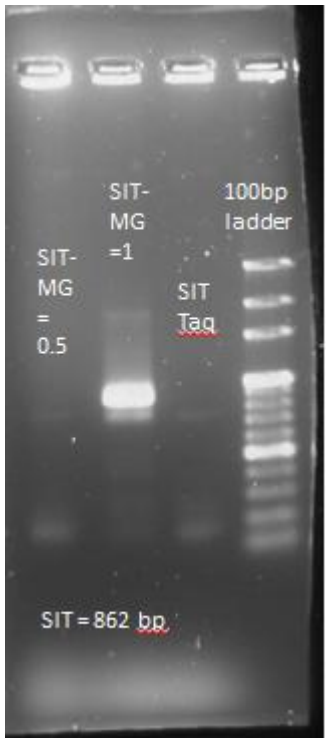
E-PR:**36.35 ng/uL**

S-PI**:54.58 ng/uL**

(2) E-PR+E-ter/ S-PI+S-ter junction PCR:

We adopted the junction primers to integrate the former assembly product (E-PR/ S-PI) each with its terminator sequence by PCR reaction.

Junction PCR →PCR RUN→Gel check

E-RT(E-PR+E-ter)	**S-IT(S-PI+S-ter)**
	

We slice the PCR products bands of each(E-RT=1128 bp/ S-IT=862bp) and do the gel extraction of each.

Concentration:

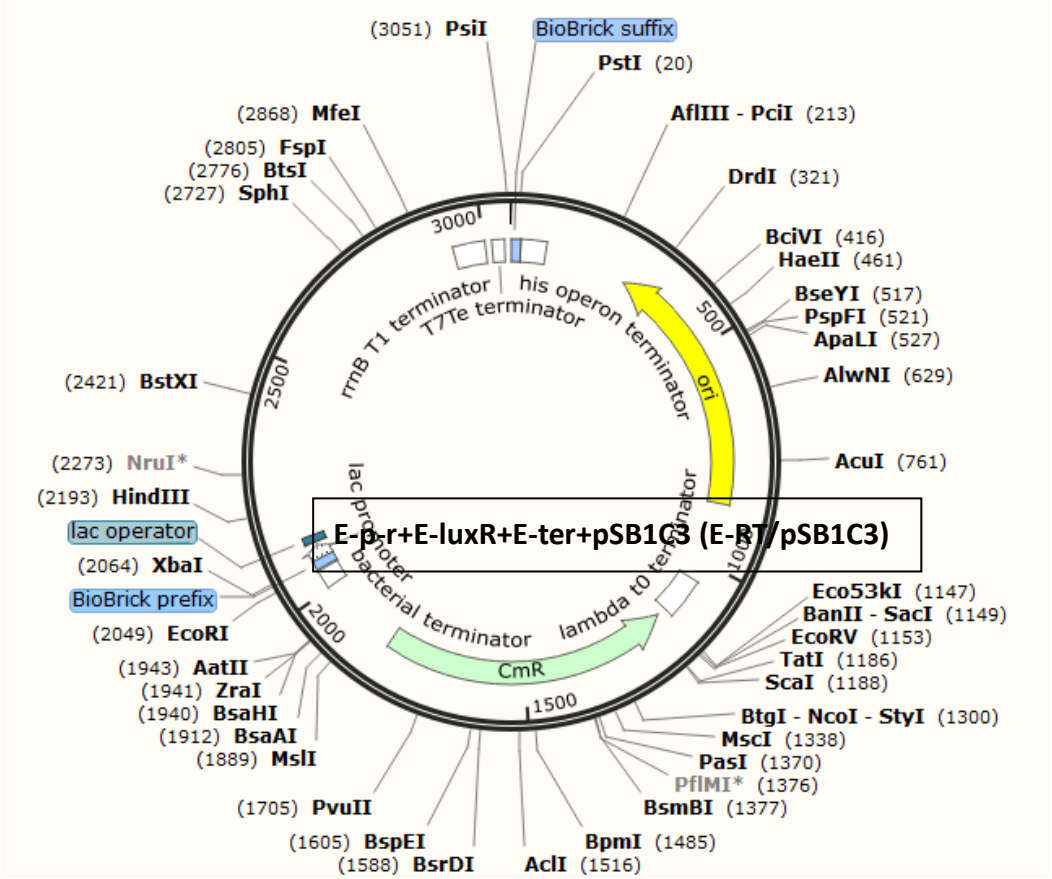
E-RT:47.02 ng/uL

S-IT:50.06 ng/uL

4. Stage four: Insert the circuit products into vector backbones

Respectively, we are planning to express our two product circuits in vector pSB1C3 (In E.coli) and PVA838 (Shuttled into S.mutans) and transform the vectors into the two organisms (E.coli and S.mutans).

(1) **E-RT + pSB1C3 ligation:**



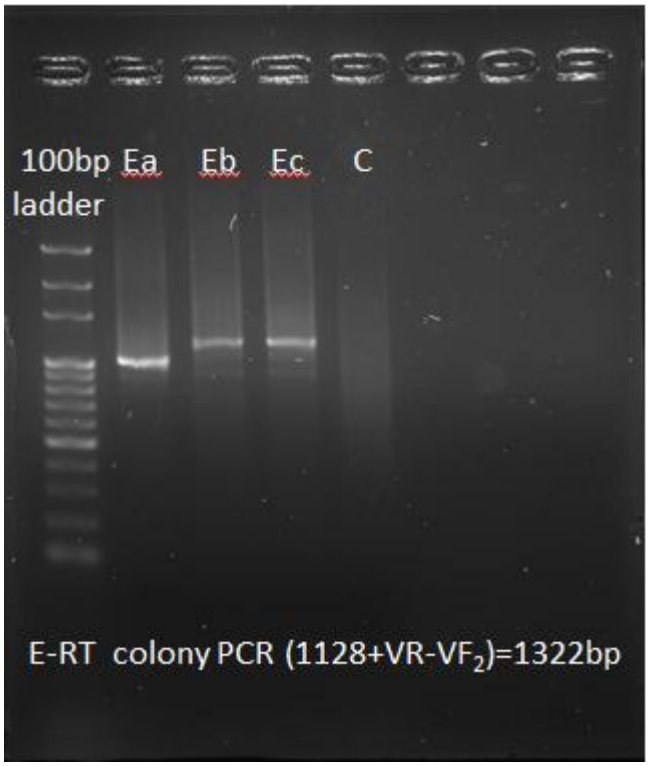
(a)Insert digestion(E-RT=47.02ng/uL): EcoR1 & Spe1

(b)Vector digestion(E1010->pSB1C3): EcoR1 & Spe1

(c)E-RT+pSB1C3 ligation:

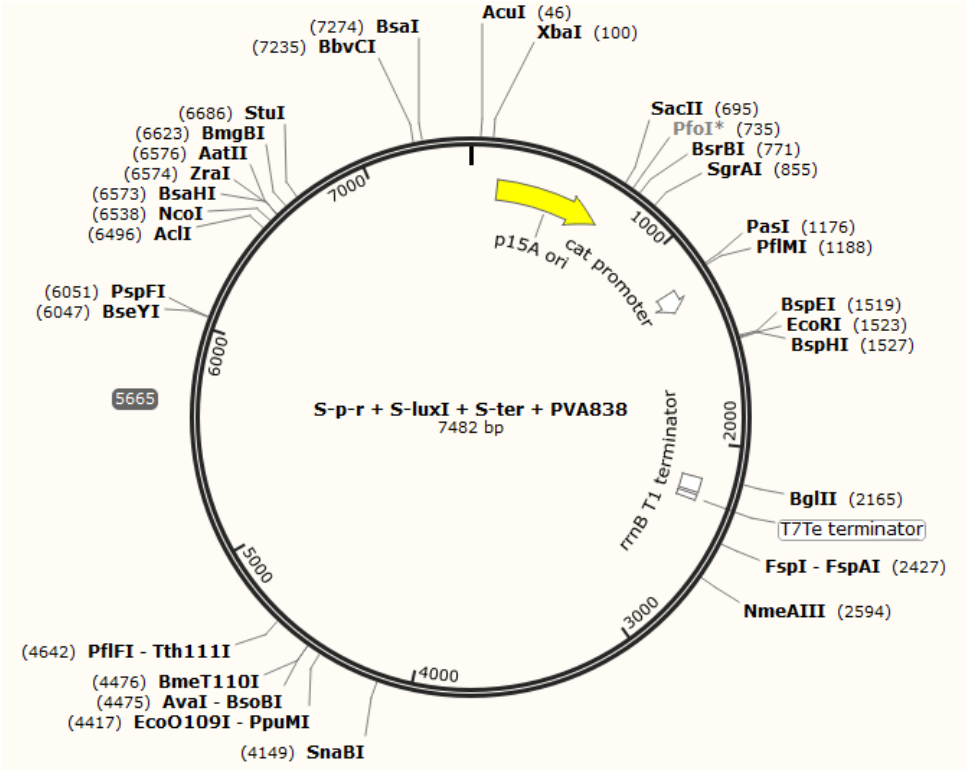
UNIT (uL)	
insert	0.7
vector	1.48
T4 ligase	0.5
10x T4 ligase buffer	1
ddH_2O	6.32

Result:



After transformation of the ligation product and O/N culture, we picked three(**Ea;Eb;Ec**) single colony and do the colony PCR using primer VR+VF2 to examine the size of the product. And the correct ones would be colony Eb and Ec that shows the size of E-RT equals to 1322bp on the agarose gel.

(2) **S-IT + PVA838 ligation:**



(a) Insert digestion(S-IT=50.06 ng/uL): EcoR1 & BamH1

(b) Vector digestion(PVA838=116.86 ng/uL): EcoR1 & BamH1

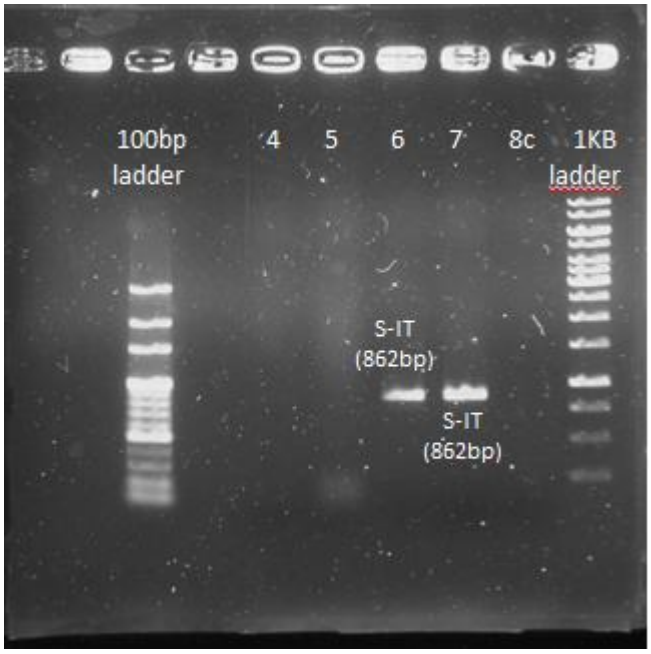
(c) S-IT+PVA838 ligation:

UNIT (uL)	
insert	0.22
vector	3.02
T4 ligase	0.5
10x T4 ligase buffer	1
ddH_2O	5.26

(d) Result(Transformed into **E.coli**):

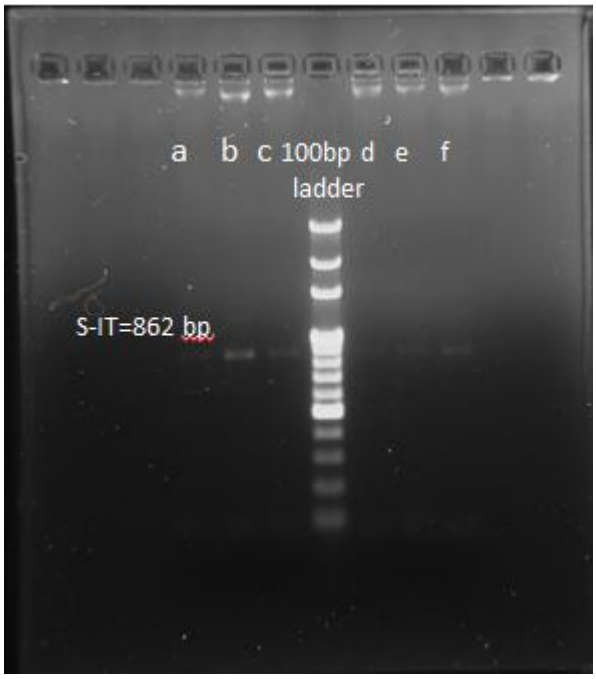
We transformed the ligation product of S-IT and shuttled vector PVA838 into E.coli competence cells and pick 4 single colonies to go through colony PCR**(primer: S-p-r Forward/ S-ter Reverse)** and check the size of S-IT.

Therefore, the colonies 6 and 7 which carry clear and correct bands would be our template colonies later transforming into S.mutans.

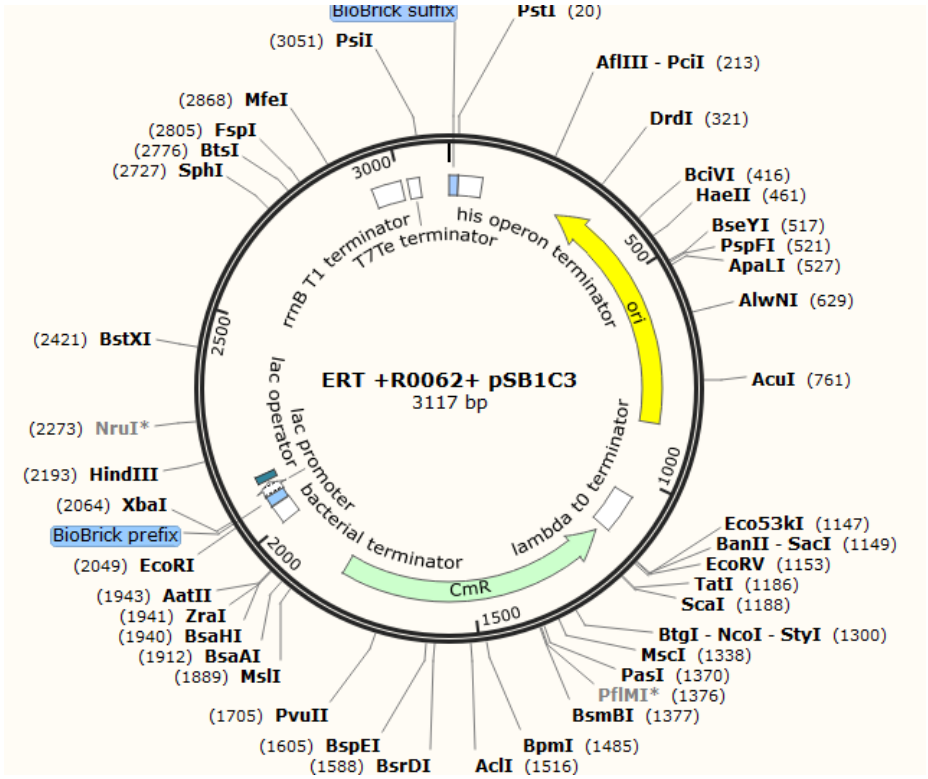


(e) Transformation of the ligation product of S-IT and PVA838 into S mutans

We extract the plasmids (construct plasmid S-IT+ PVA838) from the E.coli transformed colonies 6 and 7 mentioned above and adopted ***S.mutans transformation protocols*** *to transform the construct plasmids into S.mutans. Later on, we do colony PCR to check whether the transforming results are correct yet acquire incorrect results. Thus, we do further plasmid PCR with the inserted plasmid from the transformed S.mutans and used primers* ***(*****primer: S-p-r Forward/ S-ter Reverse*****)*** *to do plasmid PCR for re-check the size of transforming product.*
The gel electrophoresis results showed the six colonies we chose were in correct size (862bp), thus we do further liquid culture of the engineered S.mutans (colony a-f) in the ***BHI medium*** *to prepare for the functional measurements.*



(3) **Working circuit: E-RT/pSB1C3 +R0062 ligation:**



(a) Insert digestion(BBa_R0062= 76.68ng/uL): Xba1 & Pst1

(b) Vector digestion(E-RT/pSB1C3= 66.89ng/uL): Spe1 & Pst1

(c) E-RT/pSB1C3+BBa_R0062 ligation:

UNIT (uL)	
Insert(BBa_R0062)	0.12
Vector(E-RT/pSB1C3)	9.99
T4 ligase	0.5
10x T4 ligase buffer	1
ddH_2O	0

(d) We transformed the ligation product into S.mutans and growth 24hr to harvest single colonies, however, our colony PCR results was negative. Therefore, we culture the colony in liquid broth in order to extract the plasmid and test the product size by plasmid PCR using primer VR/VF2.

Reference:

1: IGEM toolkit transformation protocol: http://parts.igem.org/Help:Distribution_Kits